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Hepatitis B Virus Surface Antigen Binds to Apolipoprotein H

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We have previously demonstrated that a plasma membrane-enriched fraction isolated from human liver is capable of binding recombinant hepatitis B surface antigen (rHBsAg) (P. Pontisso, M. A. Petit, M. Bankowski, and M. E. Peeples, J. Virol. 63:1981–1988, 1989). In this study we have separated the plasma membrane proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and used a ligand-blotting technique to identify a 46-kDa rHBsAg-binding protein. This protein could be removed from the membranes with a weakly acidic buffer, implying that it is peripherally bound. Examination of human serum revealed that the 46-kDa binding protein is a serum protein. Isolation of plasma lipoproteins revealed that the binding protein is in part associated with chylomicrons and high-density lipoproteins, both of which are targeted to the hepatocyte during the normal course of lipid metabolism. The binding protein was identified as apolipoprotein H (apo H), also known as β2-glycoprotein I, on the basis of copurification of the rHBsAg-binding activity with the apo H protein and the ability of cDNA-expressed apo H to bind rHBsAg. Serum-derived HBsAg also binds to apo H, indicating that binding is not unique to rHBsAg. Binding is saturable, requires only the small S protein of rHBsAg, and is inhibited by excess rHBsAg, antibodies to HBsAg, and antibodies to apo H. The binding activity of apo H is destroyed upon reduction, indicating that 1 or more of its 22 disulfide bonds are required for interaction with rHBsAg. The possibility that an interaction between hepatitis B virus particles and lipoprotein particles may facilitate entry of the virus into hepatocytes is discussed.

Hepatitis B virus (HBV) is a member of the hepadnavirus family, which includes duck HBV, woodchuck hepatitis virus, and ground squirrel hepatitis virus. These viruses are highly infectious for their host animals, targeting primarily, though not exclusively, the liver. Cell culture systems to study hepadnavirus infection, particularly the initial steps, have required primary culture of hepatocytes (18, 66). Established cell lines derived from hepatomas have, in nearly all cases, been found to be refractory to hepadnavirus infection. However, some established cell lines are permissive for virus replication when transfected with viral DNA (11, 64). It appears, therefore, that these cell lines lack an early function(s) required for infection, a receptor for the virus, and/or another function required for entry.

HBV produces and incorporates three surface (S) proteins into its virion envelope. Because of their positions in the virion, any of these three surface proteins, or a combination thereof, could be involved in attachment of the virus to hepatocytes. The S proteins are a "nested set" of molecules encoded by a single open reading frame in the virus genome. All three have a common carboxyl terminus, but each has a unique amino terminus. The small S protein consists of only the S domain, the middle S protein contains the pre-S2 domain in addition to S, and the large S protein contains the pre-S1 domain in addition to pre-S2 and S. These proteins not only constitute the outer surface of the infectious "Dane" particle but also are the only viral components of the noninfectious hepatitis B surface antigen (HBsAg) particles.

It is not clear which of these proteins is responsible for attachment to a target cell (42). Antibodies prepared against any of the domains, pre-S1 (50), pre-S2 (22), or small S (43, 54), are able to neutralize HBV infectivity. Evidence has been presented for the involvement of pre-S1 in binding to HepG2 cells (49), as well as many other cell types (53), and in binding liver membranes (60, 61). Evidence has also been presented for the involvement of pre-S2 binding to HepG2 cells (49), to T lymphocytes (16), to polymerized human serum albumin (38), and via polymerized human serum albumin to liver plasma membranes (60). Particles containing the small S protein have also been shown to bind specifically to human hepatocytes, fibroblasts, and mononuclear blood cells (36). We have presented evidence that recombinant HBsAg (rHBsAg) containing small S, alone, is capable of binding to a primate kidney cell line (30, 31, 56).

As a step toward identifying the hepatocyte HBV receptor, we previously isolated a human liver fraction enriched in plasma membrane and demonstrated that recombinant large S protein (rLHBsAg) was capable of binding to these membranes in a specific manner (60). In this study we have further examined the human liver plasma membrane fraction in an attempt to identify an rHBsAg-binding protein(s). A ligand-blotting system was used to identify and partially characterize a 46-kDa ^{125}I-rLHBsAg-binding protein associated with liver plasma membrane, plasma, and lipoproteins. This binding protein was identified as apolipoprotein H (apo H). An enzyme immunoassay developed to characterize the properties of rHBsAg binding to apo H was used to demonstrate the specificity of this interaction.

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MATERIALS AND METHODS

Isolation of liver plasma membrane. Human cadaver livers that had been harvested for the purpose of transplantation, perfused, and stored in Viaspan (Belzer-University of Wisconsin) solution (Dupont), but not used for transplantation, were the starting material for membrane isolation. These tissues were soaked at 4°C in phosphate-buffered saline (PBS)–1 mM phenylmethylsulfonyl fluoride–0.2 U of aprotinin per ml, divided into 2.5-cm cubes as rapidly as possible, snap-frozen in liquid nitrogen, and stored at −70°C. Plasma membrane was isolated from 25 to 50 g of crude liver as described by Hubbard et al. (21). Briefly, the liver tissue was minced in cold ST buffer (0.25 M sucrose, 5 mM Tris [pH 8.0], 1 mM phenylmethylsulfonyl fluoride, 0.2 U of aprotinin per ml) and homogenized with 10 to 20 strokes of a ground glass Dounce homogenizer followed by 10 to 20 strokes of a smooth glass Dounce homogenizer. Nuclei and cell debris were removed by centrifugation at 4°C for 5 min at 250 × *g*. The supernatant was centrifuged at 4°C for 10 min at 2,000 × *g*, and the pellet was resuspended in ST buffer with the Dounce homogenizer. The density was adjusted to 1.18 g of sucrose per cm^3 with 2.0 M sucrose–5 mM Tris (pH 8.0), and the material was transferred to ultracentrifuge tubes, overlaid with ST buffer, and centrifuged at 25,000 rpm for 1 h in a Beckman SW27 rotor. The plasma membrane fraction was visualized with indirect lighting and collected from the interface between the 1.18-g/cm^3 sucrose and ST buffer layers with a Pasteur pipet. The remaining membranes were isolated from the supernatant of the 2,000 × *g* centrifugation step by pelleting this material at 25,000 rpm for 1 h. These membranes are described as internal membranes to differentiate them from the plasma membrane-enriched fraction. All membranes were washed once in 10 mM Tris buffer (pH 8.0) prior to storage at −70°C. Membrane fractions were analyzed for 5′ nucleotidase, a plasma membrane marker (33), and for glucose-6-phosphatase, an endoplasmic reticulum marker (2). Relative to the whole liver homogenate, these membranes were enriched fivefold in 5′ nucleotidase activity over glucose-6-phosphate.

HBsAg. rHBsAg containing the small S (rSHBsAg), middle S (rMHBsAg), and large S (rLHBsAg) proteins produced in *Saccharomyces cerevisiae* (14, 29, 43) and purified were gifts from R. Ellis (Merck, Sharp & Dohme Research Laboratories, West Point, Pa.). rS,L* particles, a gift from T. Rutgers (SmithKline-Biologicals, Rixenart, Belgium), were purified from *S. cerevisiae* expressing both a small S gene and a large S gene containing deletions within the pre-S regions (10). Serum-derived HBsAg (a gift from J. Gerin, Georgetown University) had been pelleted twice from sera with high concentrations of Dane particles as determined by electron microscopy and stored at −70°C (25).

rHBsAg was radiolabeled with $Na^{125}I$ (Amersham) by using Iodobeads (Pierce Chemical Co., Rockford, Ill.) according to the manufacturer's procedure. Ten micrograms of protein was labeled with 200 μCi of $Na^{125}I$. Free ^{125}I was removed on a 5-ml column of P-6DG desalting gel (Bio-Rad Laboratories) preblocked with 500 μg of bovine serum albumin (BSA) and washed extensively with PBS. Fractions containing particles were pooled and stored at 4°C for a maximum of 3 weeks before use.

Biotinylation of rS,L* particles (50 to 100 μg in 100 μl) with *N*-hydroxysuccinimide-LC-biotin was performed as described by the manufacturer (Pierce Chemical Co.). The reaction mixture was then brought to 500 μl with PBS and passed over a NAP-5 column (Pharmacia, Piscataway, N.J.), as described by the supplier, to separate free NHS-LC-biotin from biotinylated rS,L* particles. The labeled particles were then stored at −70°C until used.

Ligand-blotting assay. Protein concentrations were determined by either the Pierce or Bio-Rad protein assay with BSA as the standard. Protein from membrane fractions or serum fractions was solubilized in sodium dodecyl sulfate (SDS) sample buffer under nonreducing conditions and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (32) on 12.5% polyacrylamide gels, unless otherwise noted. Prestained molecular weight markers (Diversified Biotech) were used to calibrate protein migration. The proteins in these gels were electroblotted to nitrocellulose according to the method supplied by the apparatus manufacturer (Hoeffer Scientific Instruments). Total transferred proteins were detected by staining with Aurogold (Janssen Life Science Products) according to the manufacturer's instructions.

For ligand blotting with rHBsAg, the nitrocellulose was blocked with 5% BSA (fraction V; Sigma Chemical Co.) in PBS, pH 7.4. The blots were incubated with 10,000 to 100,000 cpm (per lane) of ^{125}I-labeled protein (5 to 50 ng) diluted in 1 ml of PBS–5% BSA for 1 h and then washed six times in PBS–0.05% Tween 20. Dried blots were exposed to preflashed X-ray film (34) for 1 to 7 days at −70°C with enhancing screens.

Alternatively, 1 ml of unlabeled rHBsAg (1 μg, unless otherwise noted) was incubated with a nitrocellulose strip. Bound rHBsAg was detected with an immunoglobulin M (IgM) monoclonal antibody (MAb), H166 (a gift from L. Mimms, Abbott Laboratories, Abbott Park, Ill.) specific for HBsAg, in PBS–5% BSA, followed by rabbit anti-mouse immunoglobulin–alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) in PBS–2% BSA–10% normal goat serum and by substrate (5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt [GIBCO]) and nitroblue tetrazolium chloride (Bio-Rad) in 0.1 M Tris-HCl (pH 9.5)–0.1 M NaCl–50 mM $MgCl_2$. As a control for the detecting antibody, an IgM mouse myeloma protein, MOPC 104 E (Sigma Chemical Co.), or an IgG MAb, P1a (55), against the P protein of Newcastle disease virus was used. Serum-derived HBsAg was incubated with blots in a similar manner, except that the blots were blocked with 1.5% gelatin for 1 h at 37°C before addition of the HBsAg, and modified TEG (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.06% SDS, 0.5% Triton X-100, and 0.25% gelatin) buffer was used to wash the blots eight times before addition of the detecting antibody. Blots were scanned with an LKB 2202 Ultroscan laser densitometer for quantification and analyzed with the Hoefer GS-365 data system.

Lipoprotein isolation. Lipoproteins were isolated from plasma by sequential flotation ultracentrifugation (63). Briefly, 4 h after a meal, blood from a healthy donor was collected into EDTA-containing tubes and centrifuged at 2,000 × *g* for 20 min to remove cells. The subsequent centrifugations were all performed with a Beckman 60 Ti rotor. The plasma was centrifuged at 20,000 rpm at 12°C for 20 min, and chylomicrons were removed from the top of the tube. The remaining fluid was centrifuged at 48,000 rpm for 20 h, and very-low-density lipoproteins (VLDL) were collected from the top of the tube. The density of the remaining solution was increased to 1.063 g/ml with NaCl and centrifuged at 48,000 rpm for 20 h, and low-density lipoproteins (LDL) were collected from the top of the tube. The high-density lipoprotein (HDL) fractions were obtained by increasing the density of the remaining solution to 1.125 and 1.21 g/ml (27) for HDL_3 and HDL_2, respectively, with NaBr and centrifugation at 48,000 rpm at 10°C for 20 h. The remaining solution was designated lipoprotein-deficient

plasma. Each of these fractions, including the lipoprotein-deficient plasma, was dialyzed against PBS.

Isolation and purification of apo H from plasma. The procedure of Polz et al. (59), with minor modifications, was used to isolate apo H. Briefly, serum proteins remaining after sequential precipitations with Rivanol (Sigma) (pH 8.0) and $HClO_4$ were lyophilized, rehydrated in 0.1 M Tris-HCl buffer (pH 8.0), and passed over a heparin-Sepharose column (Bio-Rad) which had been equilibrated with the same buffer. The column was washed with 75 ml of 0.05 M NaCl–0.002 M sodium phosphate (pH 7.4) (buffer A) until no protein could be detected in the eluate. The column was sequentially eluted with buffer A alone and with buffer A containing 0.2, 0.35, and 0.5 M NaCl. The eluate following each buffer addition was lyophilized, resuspended in PBS, and dialyzed against PBS. The protein concentration of each fraction was analyzed with the Bio-Rad protein assay.

For the ligand blot experiments other than the one described above, apo H was purified by the method of McConathy and Alaupovic (44). $HClO_4$-soluble proteins were applied to a Toyo Soda DEAE 5PW column and eluted with an NaCl gradient prepared in 50 mM Tris-HCl (pH 8.0). Fractions containing apo H, as determined by SDS-PAGE, were pooled, lyophilized, rehydrated in water, and applied to a Toyo Soda TSK G-3000 SW gel filtration column equilibrated with 0.2 M ammonium formate. The column was eluted with the same buffer, and fractions containing apo H were pooled, lyophilized, dissolved in PBS, and dialyzed against PBS. Both methods produced highly purified apo H, as determined by Coomassie brilliant blue staining of SDS-PAGE-separated proteins.

Transient expression of apo H in COS-1 cells. COS-1 cells were transfected with a eukaryotic expression vector, pRc/G2ApoH, containing a cDNA clone of the apo H gene (45) by the DEAE-dextran method (46). pRc/G2apoH was a gift from M. Nunn (Cytel Corporation). At 48 h after transfection, cells were washed and incubated for an additional 4 h in serum-free medium. The supernatant from these cells was cleared of debris by centrifugation at 2,000 × *g* before analysis.

Enzyme immunoassay. MAb P2D4 against apo H was raised in RBF/DnJ mice. Antibodies were purified from tissue culture supernatants on protein G agarose. Antiserum to apo H was generated by subcapsular immunization of a rabbit with 150 μg of purified apo H isolated by the method of McConathy and Alaupovic (44) and emulsified in complete Freund's adjuvant (Difco). One month later the rabbit received a similar immunization with apo H in incomplete Freund's adjuvant. Ten days later the rabbit was bled and serum was isolated.

In the capture enzyme immunoassay, 50 μl of MAb P2D4 (36 μg/ml) in PBS was adsorbed to vinyl 96-well assay plates (Costar, Cambridge, Mass.) by overnight incubation at 4°C with a cover to prevent evaporation. The wells were washed three times with PBS (the wash procedure used between all additions described below) and blocked with PBS containing 1% BSA (the diluent used for all subsequent additions) at 37°C for 1.5 h. Purified apo H (50 μl) was diluted and incubated in the antibody-coated wells for 1.5 h at 37°C. To detect bound apo H, diluted rabbit anti-apo H (50 μl), goat anti-rabbit IgG-linked alkaline phosphatase (50 μl) (Kirkegaard & Perry Laboratories, Inc.), and 100 μl of *para*-nitrophenyl phosphate substrate (Kirkegaard & Perry Laboratories, Inc.) were added sequentially. The intensity of color was quantified at 405 nm with a Titertek Multiskan Plus (ICN/Flow Laboratories).

To examine its binding activity, apo H (80 ng/ml) was added to MAb P2D4-coated microtiter wells and incubated with biotinylated rS,L* particles for 16 h at 22°C. Bound particles were detected by streptavidin-alkaline phosphatase (Bethesda Research Laboratories, Bethesda, Md.) followed by substrate. In some experiments, unlabeled rS,L* particles were used. In those cases, bound rS,L* particles were detected by goat anti-HBs antiserum (DAKO Corporation, Carpinteria, Calif.) followed by rabbit anti-goat IgG–alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc.) followed by substrate.

Reagents. Trypsin (from bovine pancreas, type 13), aprotinin, and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co.

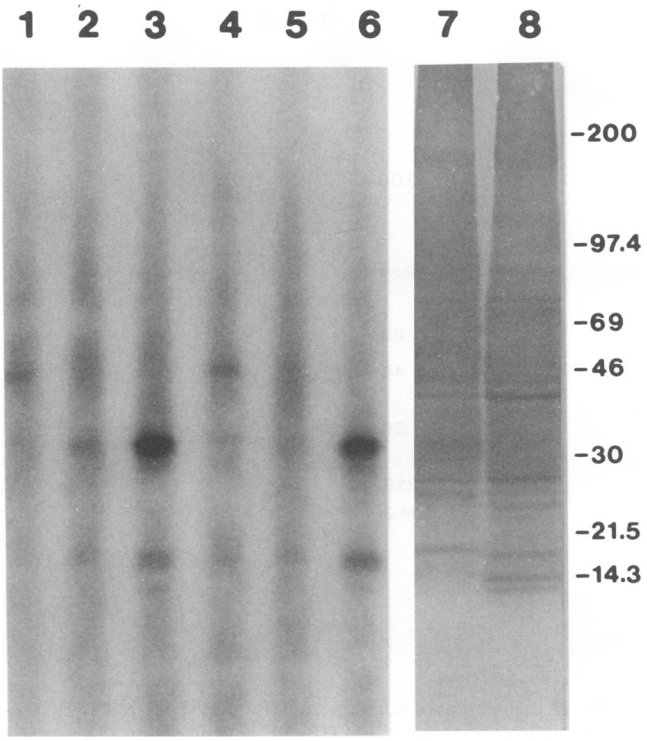

FIG. 1. Ligand blot of human liver membrane fractions separated by electrophoresis and probed with ^{125}I-rLHBsAg. Seventy-five-microgram portions of the human liver plasma membrane-enriched fraction, internal membrane, and crude lysate were displayed on a 5 to 20% gradient polyacrylamide gel. Two separate preparations (lanes 1 to 3 and lanes 4 to 6) were examined. Lanes 1 and 4, plasma membrane-enriched fractions; lanes 2 and 5, the remaining, internal membrane fractions; lanes 3 and 6, starting liver homogenate; lanes 7 and 8, total proteins detected with Aurogold staining from the plasma membrane fraction in lane 1 and from the lysate in lane 3, respectively. Numbers on the right are molecular masses in kilodaltons.

RESULTS

Human liver plasma membrane proteins probed with rLHBsAg. We previously found that rLHBsAg binds to a plasma membrane-enriched fraction isolated from human liver (60). In an attempt to determine the molecular weight of the molecule in this fraction which binds rLHBsAg, a ligand-blotting procedure was used. The plasma membrane-enriched fraction was solubilized in SDS-containing sample buffer, electrophoresed on an SDS-polyacrylamide gel, electroblotted to nitrocellulose, and probed with ^{125}I-rLHBsAg. A single major species of 46 kDa bound ^{125}I-rLHBsAg (Fig. 1, lanes 1 and 4). To determine whether this species was unique to the plasma membrane-enriched fraction, equivalent amounts of

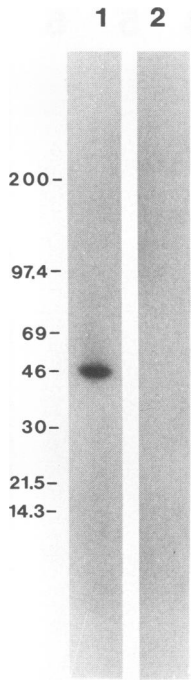

FIG. 2. Ligand blot of human serum proteins separated by electrophoresis and probed with ^{125}I-rLHBsAg. Human serum (75 μg) was dissociated in sample buffer without (lane 1) or with (lane 2) 10% β-mercaptoethanol, electrophoresed on an SDS–15% polyacrylamide gel, electroblotted to nitrocellulose, and probed with ^{125}I-rLHBsAg. Numbers on the left are molecular masses in kilodaltons.

protein from the internal membrane fraction and the crude liver homogenate were also probed in this ligand blot. The 46-kDa binding protein was not detected in the crude lysate (Fig. 1, lanes 3 and 6), indicating that it is in a low concentration in the tissue. The lysate, and to a lesser extent the internal membranes (Fig. 1, lanes 2 and 5), displayed binding activity at approximately 15 and 32 kDa. Since these proteins are not in the proper position in the cell to act in the initial binding of the virus to the cell, they were not studied further.

Pretreatment of the plasma membrane-enriched fraction with trypsin or a reducing agent prior to electrophoresis destroyed the 46-kDa binding activity (24). Unexpectedly, the 46-kDa binding activity was also lost by treatment of the plasma membrane-enriched fraction with a mildly acidic buffer (PBS with 10 mM acetic acid added) followed by centrifugation to collect the membranes (24). This type of treatment removes peripheral membrane proteins and proteins bound to receptors (7). The binding activity of the 46-kDa protein per se is not sensitive to low pH, as indicated by the purification procedure described below, which includes a low-pH step. The 46-kDa protein, therefore, is a peripheral or receptor-bound protein.

The 46-kDa binding protein in serum. Since peripheral or receptor-bound proteins on liver plasma membranes would probably come from circulating blood, human serum proteins were examined for their ability to bind ^{125}I-rLHBsAg. ^{125}I-rLHBsAg bound strongly to a 46-kDa species in human serum (Fig. 2, lane 1). If the serum was reduced before electrophoresis, ^{125}I-rLHBsAg-binding activity was lost (Fig. 2, lane 2). The plasma binding protein and the plasma membrane-associated binding protein appear to represent the same protein, since they

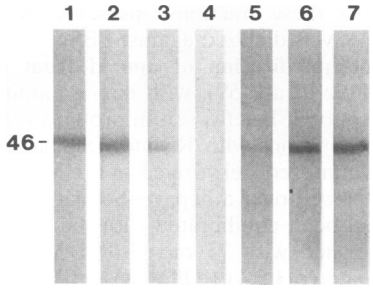

FIG. 3. Ligand blot of serum lipoprotein fractions probed with ^{125}I-rLHBsAg. One hundred micrograms of protein from each fraction was displayed by SDS-PAGE, electroblotted to nitrocellulose, and probed with ^{125}I-rLHBsAg. Lanes: 1, total plasma; 2, chylomicrons; 3, VLDL; 4, LDL; 5, HDL_2; 6, HDL_3; 7, lipoprotein-free plasma. The position of the 46-kDa protein is indicated.

migrate identically upon electrophoresis and the rLHBsAg-binding activities of both proteins are sensitive to reduction.

As a first step in fractionating human plasma to identify the 46-kDa binding protein, lipoprotein particles were isolated. Fractions containing chylomicrons, VLDL, LDL, HDL_2, and HDL_3 were isolated by sequential density increases and ultracentrifugation and analyzed in the ligand blot with ^{125}I-rLHBsAg (Fig. 3). A large amount of 46-kDa binding protein was found in the chylomicron and HDL_3 fractions (Fig. 3, lanes 2 and 6, respectively); the VLDL and HDL_2 fractions displayed less binding protein (lanes 3 and 5, respectively), and the LDL fraction (lane 4) did not contain detectable binding protein. Lipoprotein-deficient plasma remaining after the lipoprotein isolation procedure also contained the 46-kDa binding protein (Fig. 3, lane 7), as did the plasma starting material (lane 1).

Binding of HBsAgs with different compositions. Initial experiments in which ligand blots were probed with similar amounts of ^{125}I-rSHBsAg or ^{125}I-rMHBsAg detected no binding to any serum protein (67). However, we discovered that these rHBsAg preparations were inefficiently labeled with ^{125}I, suggesting that these results might not be directly comparable to those obtained with ^{125}I-rLHBsAg. In fact, when 10-fold more ^{125}I-rSHBsAg or ^{125}I-rMHBsAg was added to a blot, binding to the 46-kDa protein could be detected (67).

To avoid problems with unequal ^{125}I labeling, a different system was devised to detect rHBsAg binding. Unlabeled rHBsAg was incubated with the blot, and bound particles were detected with MAb H166, against the small S protein of HBsAg, followed by alkaline phosphatase-labeled secondary antibody. In addition to rSHBsAg, rMHBsAg, and rLHBsAg, rS,L* particles (10) were used. The rS,L* particles contain both the small S protein and a deleted form of the large S protein which has retained the peptide suggested to contain the hepatocyte-binding region (49). With this detection system, rLHBsAg, rMHBsAg, rSHBsAg, and rS,L* were all able to bind the 46-kDa protein from serum (Fig. 4A, lanes 2, 4, 6, and 8, respectively). Therefore, the pre-S sequences are not required for binding to the 46-kDa protein; the small S protein is sufficient. This conclusion is confirmed by competition experiments whose results are presented below.

Serum-derived HBsAg was tested for its ability to bind to this 46-kDa protein. As shown in Fig. 4B, HBsAg isolated from the sera of two individuals bound to the 46-kDa protein (lanes 2 and 4), as did the rS,L* particles. The serum-derived HBsAg preparations also bound to high-molecular-weight material. This interaction may be due to serum proteins associated with these HBsAg.

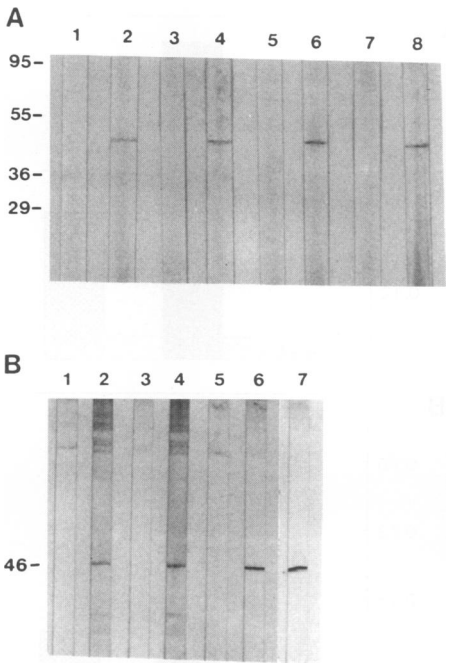

FIG. 4. rHBsAg and serum-derived HBsAg binding to the 46-kDa serum protein. Plasma (1 μl) was displayed by SDS-PAGE and electroblotted to nitrocellulose. (A) Strips of nitrocellulose were incubated with rLHBsAg (lanes 1 and 2), rMHBsAg (lanes 3 and 4), rSHBsAg (lanes 5 and 6), or rS,L* (lanes 7 and 8). Lanes 1, 3, 5, and 7 were then incubated with irrelevant mouse IgM, while lanes 2, 4, 6, and 8 were incubated with MAb H166, specific for the small S protein of HBsAg. (B) Strips of nitrocellulose were incubated with 250 ng of serum-derived HBsAg preparation I (lanes 1 and 2) or II (lanes 3 and 4) or with rS,L* particles (lanes 5 and 6). Lanes 1, 3, and 5 were then incubated with irrelevant mouse IgM. Lanes 2, 4, and 6 were incubated with MAb H166. Lane 7 was incubated with MAb P2D4, which recognizes apo H. Numbers on the left are molecular masses in kilodaltons.

Identification of the 46-kDa protein. Plasma lipoprotein particles contain three 46-kDa proteins: a disulfide-linked heterodimer of apo E and apo A-II, apo A-IV (41), and apo H (44). Neither the apo E–apo A-II heterodimer nor apo A-IV (kind gifts from K. Weisgraber, University of California, San Francisco) was able to bind ^{125}I-rLHBsAg (1). The remaining possible protein, apo H, has been shown to be associated with lipoproteins, particularly with chylomicrons and HDL, as well as being found free in the serum (35, 58). This pattern is similar to that found for the 46-kDa rHBsAg-binding protein (Fig. 3).

To determine whether the 46-kDa protein is apo H, apo H was purified by a standard technique and tested for its ability to bind rS,L* particles. Apo H was purified from human plasma on the basis of its acid solubility and on its ability to bind tightly to heparin (59). A 46-kDa doublet was found in the final apo H-enriched fraction (Fig. 5A, lane 5) when the gel was stained with Coomassie brilliant blue. This fraction also contained the 46-kDa rS,L*-binding activity (Fig. 5B, lane 5). By quantifying the rS,L* particle binding and adjusting for the amount of protein loaded in each lane, as described in the legend to Fig. 5, the specific activity of rS,L* particle binding was found to increase 57-fold after purification. It is possible that the presence of other plasma proteins in lane 1 interfered with transfer or binding activity of the plasma apo H, resulting in an

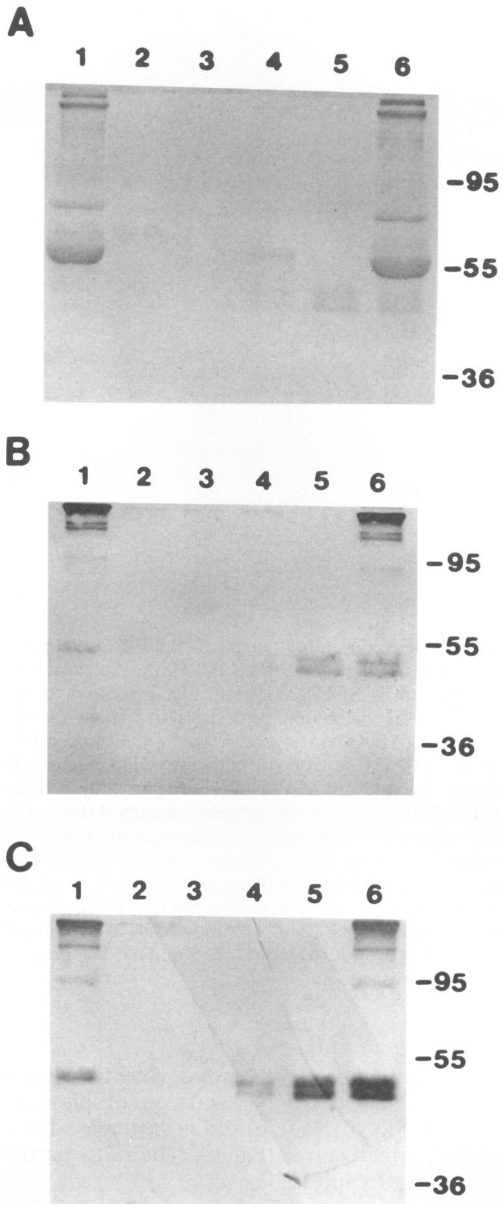

FIG. 5. Apo H purified from human plasma retains rS,L* particle-binding activity. Acid-soluble proteins from human plasma were fractionated on a heparin-Sepharose column, and the fractions were analyzed in three parallel SDS-polyacrylamide gels. One gel (A) was stained with Coomassie brilliant blue. The remaining two gels were electroblotted to nitrocellulose and incubated with rS,L* particles followed by MAb H166 to the small S protein of HBsAg (B) or with rabbit antiserum to apo H (C). In each gel, lane 1 contains plasma (26 μg of protein) and lanes 2 to 5 each contain 0.8 μg of protein from fractions collected from the heparin-Sepharose affinity column after washing with 0.05 M NaCl (lanes 2 and 3), 0.2 M NaCl (lane 4), and 0.35 M NaCl (lane 5). Lane 6 contains a mixture of 26 μg of plasma and 0.8 μg of the 0.35 M NaCl wash. Compared with plasma (lane 1), the purified apo H (lane 5) bound 1.8 times more rS,L* particles (B) and 4.9 times more rabbit anti-apo H (C), as determined by laser densitometry (average of three readings). After adjustment for the different amounts of protein loaded in lanes 1 and 5, the rS,L* particle-binding activity of the purified apo H is 57-fold greater than that of the plasma. Apo H, as detected by the antibody, was enriched by 158-fold. Numbers on the right are molecular masses in kilodaltons.

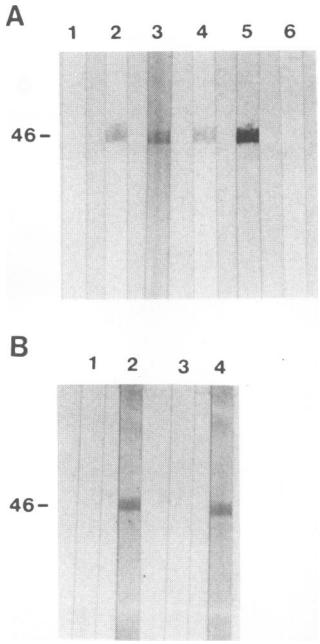

FIG. 6. rHBsAgs containing each of the surface proteins and serum-derived HBsAg bind to purified apo H. Purified apo H (1 μg per lane) was displayed by SDS-PAGE and electroblotted to nitrocellulose. (A) Strips were incubated with rLHBsAg (lane 2), rMHBsAg (lane 3), rSHBsAg (lane 4), or rS,L* (lane 5). Bound rHBsAg was detected with MAb H166. Control lanes were incubated with rLHB sAg and then with irrelevant MAb P1a (lane 1) or without rHBsAg and then with MAb H166 (lane 6). The amount of each rHBsAg added was adjusted to represent approximately equivalent reactivities with H166, as determined by enzyme immunoassay: rLHBsAg, 1.0 μg; rMHBsAg, 0.25 μg; rSHBsAg, 0.17 μg; and rS,L*, 0.25 μg. (B) Strips were incubated with 1 μg of serum-derived HBsAg preparation I (lanes 1 and 2) or II (lanes 3 and 4), followed by an irrelevant mouse IgM (lanes 1 and 3) or MAb H166 (lanes 2 and 4). The position of the 46-kDa protein is indicated.

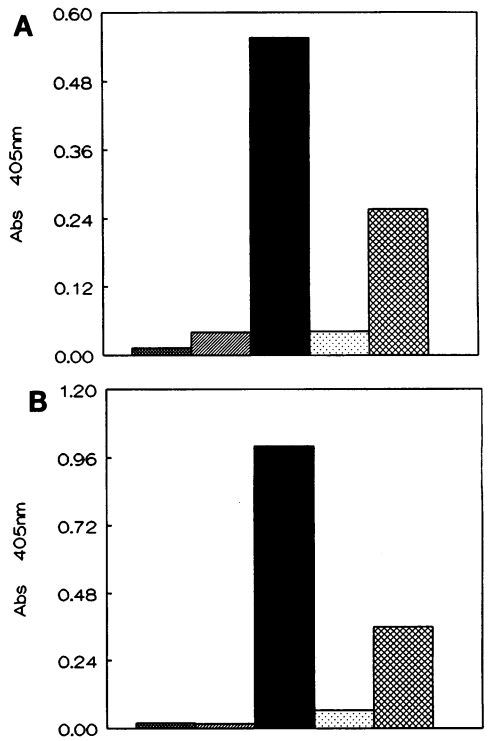

FIG. 7. Production of apo H by COS-1 cells transfected with the apo H cDNA and by HepG2 cells and the ability of that apo H to bind rS,L* particles. Microtiter wells coated with MAb P2D4 were used to capture apo H from cell culture medium lacking serum. (A) Apo H was detected by a rabbit anti-apo H serum. (B) rS,L*-binding activity was detected by incubation of all wells with rS,L* particles followed by goat anti-HBs. ▩, No antibody; ▨, medium; ■, medium from HepG2 cells; ▩, medium from COS-1 cells; ▩, COS medium from transfected COS-1 cells.

artificially low estimate of the binding activity in the unfractionated plasma. However, the addition of plasma to the purified apo H (Fig. 5B, lane 6) did not decrease the binding activity of the purified apo H (lane 5). The rS,L* particles are, therefore, binding to apo H.

Rabbit antiserum prepared against purified apo H reacted with apo H in the immunoblot (Fig. 5C, lane 5), as expected. By using this antiserum to quantify the apo H in the plasma starting material and in the purified apo H, a 158-fold purification of apo H was calculated. A comparison of the rS,L*-binding activity with the amount of apo H in the purified preparation indicates that 2.8-fold more apo H protein than binding activity was recovered, suggesting that some of the binding activity is lost during purification.

It is not clear why plasma apo H migrates as a single species (Fig. 5C, lane 1) but resolves into two species after purification (lane 5). It was possible that the single species detected in plasma is an artifact of protein overloading in that lane. This is not the case, since the two species of purified apo H are still detected (Fig. 5C, lane 6) when mixed and electrophoresed with the same amount of plasma protein as in lane 1. This result suggests that the additional, faster-migrating species of purified apo H is probably generated during purification, most likely during the acid precipitation step.

To confirm that HBsAgs of different compositions bind to apo H, purified apo H was electrophoresed, transferred, and used in a ligand blot. The amount of each rHBsAg added was normalized by its reactivity with MAb H166, the antibody used to detect HBsAg on the blot. As shown in Fig. 6A, rLHBsAg, rMHBsAg, rSHBsAg, and rS,L* all bound to purified apo H under these conditions (lanes 2 to 5, respectively), although the intensity of the signal differed among rHBsAg preparations. The rS,L* particles were the most efficient at binding and were used in all subsequent experiments. In addition to the rHBsAg preparations, two preparations of serum-derived HBsAg were tested for binding. These preparations, which were shown in Fig. 4B to bind to the 46-kDa serum protein, were also shown to bind to purified apo H (Fig. 6B, lanes 2 and 4).

Binding to recombinant apo H. Upon identifying apo H as an HBsAg-binding protein, we cDNA cloned the apo H transcript from human liver and from the human hepatoma cell line HepG2 (45). To determine whether recombinant apo H is capable of binding rS,L* particles, recombinant apo H was produced by transient expression from plasmid pRc/G2apoH in COS-1 cells incubated in serum-free culture medium.

The culture medium was analyzed for apo H in a capture assay. For this purpose, we prepared a MAb against apo H, P2D4. A constant amount of MAb P2D4 was bound to microtiter wells which were then incubated with culture medium from transfected COS-1 cells. Captured apo H was detected with the rabbit antiserum to apo H (Fig. 7A). With this method, apo H was found in the medium from the

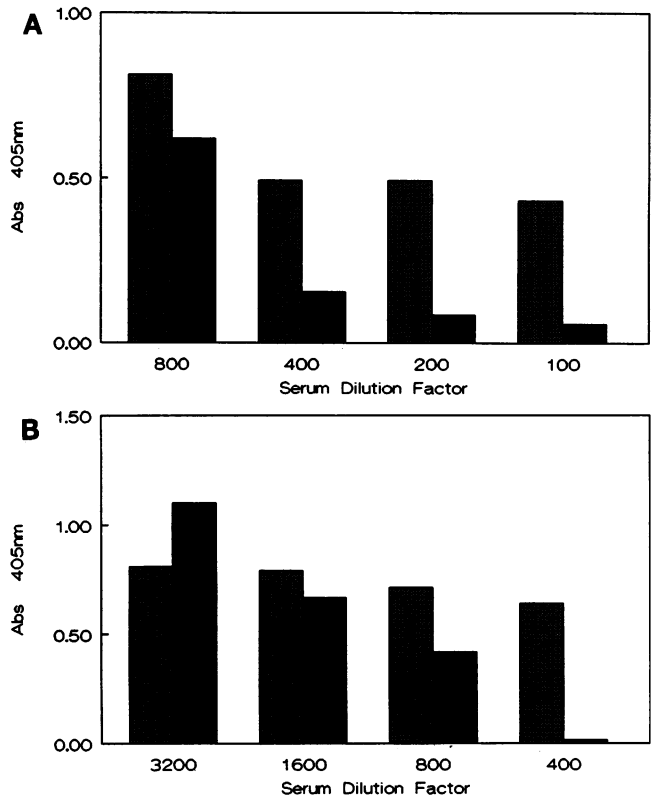

FIG. 8. Inhibition of binding of rS,L* particles to apo H by antisera against HBsAg and antisera against apo H. Microtiter wells coated with MAb P2D4 were used to capture purified apo H. Biotinylated rS,L* particles were mixed with various dilutions of goat anti-HBs (A, solid bars) or rabbit anti-apo H (B, solid bars) serum and incubated for 6 h at 22°C before being added to the microtiter wells. Normal goat sera or rabbit prebleed sera were included as controls (shaded bars).

transfected COS-1 cells but not in the medium from control COS-1 cells. These results are consistent with a previous experiment in which rabbit anti-apo H serum immunoprecipitated the anticipated 46-kDa protein from the medium of metabolically [^{35}S]methionine-labeled, transfected COS-1 cells (45).

Apo H was also detected in the medium from HepG2 cells but not in medium before incubation with these cells (Fig. 7A), indicating that HepG2 cells naturally produce apo H. In addition, apo H mRNA has been detected both in transfected COS-1 cells and in HepG2 cells but not in control COS-1 cells (45).

To determine whether recombinant apo H is capable of binding rS,L* particles, a similar experiment was performed, except that following incubation of MAb P2D4-coated wells with culture medium, rS,L* particles were added. As shown in Fig. 7B, apo H produced by the transfected COS-1 cells, as well as that produced by HepG2 cells, was able to bind rS,L* particles. These results confirm that apo H is the 46-kDa, rS,L* particle-binding protein.

Specificity of binding to apo H. If rS,L* particle binding to apo H is specific, antibodies to HBsAg and antibodies to apo H should be able to block the interaction. To test this prediction, apo H purified from serum was captured on a microtiter well by MAb P2D4. Wells were then incubated with biotinylated rS,L* particles in the presence of antiserum to HBsAg or antiserum to apo H. Both anti-HBs (Fig. 8A) and anti-apo H

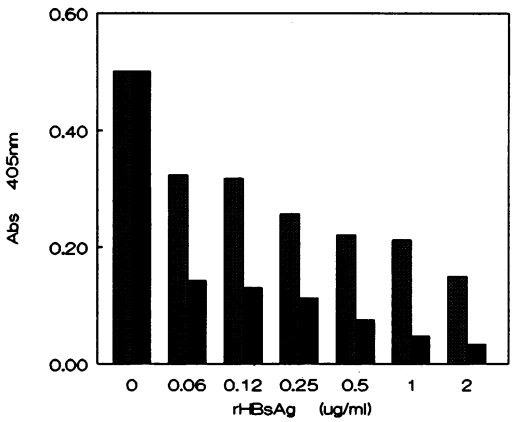

FIG. 9. Competition for rS,L* particle binding to apo H by rHBsAg particles. Microtiter wells coated with MAb P2D4 were incubated with purified apo H and then incubated for 16 h with a mixture of biotinylated rS,L* particles (shaded bars) and competing rSHBsAg particles (solid bars). The concentration of competing particles added is indicated at the bottom. Bound biotinylated rS,L* particles were detected by streptavidin-alkaline phosphatase followed by substrate.

(Fig. 8B) were able to block rS,L* particle binding to apo H, suggesting a specific interaction.

Specific binding should also be inhibited by the addition of excess amounts of rHBsAg. To test this, MAb P2D4-captured apo H on microtiter wells was incubated with biotinylated rS,L* particles in the presence of excess unlabeled rS,L* (Fig. 9). Unlabeled rS,L* was able to compete for biotinylated rS,L* binding, indicating a specific interaction. As described above, all rHBsAg preparations tested, regardless of their pre-S protein content, were able to bind to apo H (Fig. 4 and 6), leading to the suggestion that the small S protein, alone, is responsible for this interaction. To test this idea, excess rSHBsAg was mixed with biotinylated rS,L* and added to the captured apo H (Fig. 9). rSHBsAg particles efficiently com-

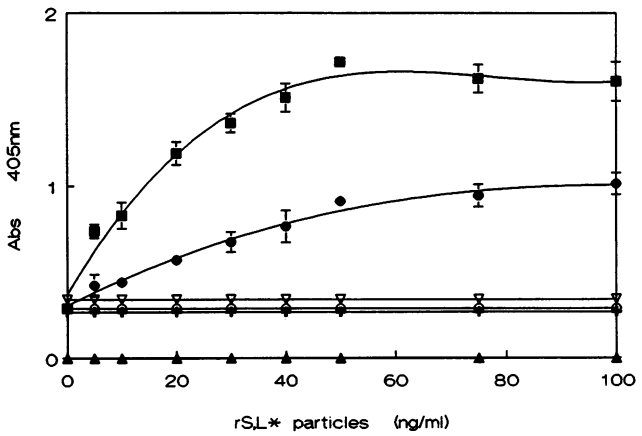

FIG. 10. Saturation of rS,L* particle binding to a constant amount of apo H. Microtiter wells coated with MAb P2D4 were incubated with 1 (●) or 3 (■) ng of purified apo H per ml. Increasing amounts of rS,L* particles were then added, and bound particles were detected with goat anti-HBs. Controls included no capture MAb (▲), no apo H (○), no rS,L* particles (+), and normal goat serum instead of goat anti-HBs (▽).

peted with rS,L* binding, confirming the idea that only small S is required for binding to apo H.

Specific binding would also be expected to be saturable. To examine this, a fixed amount of purified apo H, 1 or 3 ng/ml, was added to MAb P2D4-coated microtiter wells. Increasing amounts of rS,L* particles were then added, and bound particles were detected with goat anti-HBs. As shown in Fig. 10, rS,L* binding to both concentrations of captured apo H appeared to be saturable. An alternate explanation for these results would be that the detecting antibodies were depleted. While this might be the case in the 3-ng/ml apo H curve, it could not explain the lower, though still saturated, 1-ng/ml curve. Furthermore, a similar experiment in which the amount of apo H was increased while the amount of rS,L* was kept constant resulted in an increasing signal (47), indicating that the detection system had a wider range than that used here.

By using the approximate amount of rS,L* particles (50 ng/ml, 0.05 ml) found to saturate this amount of apo H (1 ng/ml, 0.05 ml), it was calculated that rS,L* particles (approximately 3×10^6 g/mol) bound to apo H (3.6×10^4 g/mol) in an approximate molar ratio of one rS,L* particle per two apo H molecules. This ratio indicates efficient binding between these two molecules under these conditions.

DISCUSSION

We had previously demonstrated that the HBV large S protein in the form of rLHBsAg is able to bind in a specific manner to a human liver membrane fraction enriched for plasma membranes (60). In the present report we have demonstrated by ligand blotting that this plasma membrane-enriched fraction contains a 46-kDa protein which binds ^{125}I-rLHBsAg. We have identified this binding protein as apo H by copurification of the binding activity with the apo H protein and by rS,L* particle binding to apo H produced by transient expression of the apo H cDNA. Binding is not restricted to recombinant particles, since serum-derived HBsAg also binds to apo H. Binding is specific as determined by its inhibition by antiserum to HBsAg, antiserum to apo H, or excess rHBsAg. In addition, binding is saturable and highly efficient.

Our original work with plasma membranes derived from human liver suggested that the large S protein was required for binding (60). This conclusion was consistent with that of Neurath et al. (49), who demonstrated that high concentrations of a synthetic peptide representing amino acids 21 to 47 of the preS1 region of the large S protein could inhibit binding of HepG2 cells to HBsAg linked to cellulose. This group has also found binding activity present on many other, though not all, cell types (53). Our initial work with apo H also suggested that only rHBsAg containing the large S protein bound to apo H. However, when the detection system was improved, we found that rHBsAg containing small S and middle S also bound to apo H. In previous work, using rSHBsAg, we had found that cultured hepatoma cells did not bind these particles efficiently but that Vero, African green monkey kidney cells did (56). It remains possible that the small S protein contains the HBV attachment site.

Apo H is a glycoprotein with four N-linked carbohydrate chains (45, 65) present at concentrations of approximately 200 μg/ml in serum (58). The 326-residue mature protein is composed of a 61-amino-acid motif repeated four times, followed by one longer, modified repeat. Each of the first four repeats contains four cysteines in conserved positions, linked in a 1-3 and 2-4 pattern (5, 26, 37), at least one of which is critical for its rHBsAg-binding activity. This pattern of disulfide bonds is similar to the "short consensus repeat" units found in a family of approximately 20 proteins which includes many of the complement control proteins (28). It is interesting that the Epstein-Barr virus receptor, complement receptor 2 (15), and the measles virus receptor, CD46 (13, 48), also belong to this short consensus repeat family.

We have demonstrated that rHBsAg binds to apo H, but we have not yet demonstrated the physiological relevance of this binding. Likewise, evidence for several other binding proteins as candidate receptors for HBV has been presented. Neurath et al. (49) pointed out that the portion of pre-S1 that they had identified as being involved in attachment to HepG2 cells had similarities to a portion of the IgA heavy chain, and Pontisso et al. (62) presented evidence that an IgA receptor may be involved in binding. Neurath et al. (51, 52) have reported that serum-derived HBsAg particles bind to interleukin 6, including a cell-associated form of interleukin 6, which could be involved in attachment. Franco et al. (16) have presented evidence that HBsAg binds to T cells via the transferrin receptor and have suggested that a similar binding may occur on hepatocytes. Hertogs et al. (20) have recently reported that HBsAg containing only small S binds to a 35-kDa liver protein, which they identified as endonexin II. Endonexin II has calcium channel activity (6) and is thought to be located primarily intracellularly (9). We detected a major rHBsAg-binding protein of similar size (32 kDa) in lysates of human liver, but this protein was not associated with the plasma membrane-enriched fraction (Fig. 1).

In addition, several proteins, unidentified except for their molecular masses have been suggested as receptor candidates. Petit et al. (57) identified several HepG2 cell proteins with apparent molecular masses of 35, 40, 43, and 50 kDa by using an anti-idiotype antiserum. This serum was raised against a MAb that recognizes the pre-S1 domain identified by Neurath et al. (49) as being involved in HBsAg binding to these cells. Dash et al. (12) directly used a peptide representing this domain (amino acids 21 to 47) to identify a 31-kDa binding protein by cross-linking and by affinity chromatography. Budkowska et al. (8) have identified an HBsAg-binding factor in human serum by its interference with the pre-S1- and pre-S2-specific MAb binding. This molecule has an apparent molecular mass of 50 kDa by high-pressure liquid chromatography, similar to that of apo H analyzed by SDS-PAGE. However, it appears to differ from apo H in its isoelectric point, which is 7.1, compared with the reported 5.1 to 6.1 for the five resolvable species of apo H (17). Furthermore, the ability of this protein to bind HBsAg is sensitive to either heat or SDS treatment, while that of apo H is not.

The work described in the present report was begun with the goal of identifying the cell receptor for HBV. Of the 10 or more mammalian virus receptors that have been clearly identified by cloning and expression, all are integral membrane proteins. Although we initially found apo H associated with the human liver plasma membrane fraction, it behaved as a peripheral or receptor-bound protein, removed from the plasma membrane fraction by a weakly acidic buffer.

Since apo H is associated with lipoproteins, particularly chylomicrons and HDL (35, 58) (Fig. 4), it is possible that HBV binds to apo H on the surfaces of these lipoprotein particles. In the bloodstream, chylomicrons are partially degraded by lipoprotein lipase, resulting in chylomicron remnants, which are taken up by hepatocytes (39). HDLs are also taken up by hepatocytes in the process of "reverse cholesterol transport" (23, 39). The apo H which copurified with the liver plasma membrane fraction may have been associated with specifically bound lipoproteins. It is tempting to speculate that

infectious HBV might bind to chylomicron or HDL particles and be taken into hepatocytes as a "hitchhiker," along with these lipoproteins, via the LDL receptor (40) or the LDL receptor-related protein (4).

If HBV infection of hepatocytes involves apo H on chylomicrons and/or on HDL, this would be a novel mechanism for virus attachment and entry. It might also explain why it has been so difficult to infect hepatoma-derived cells in culture. Only Bchini et al. (3) have successfully infected HepG2 cells, using very high concentrations of virus. Even infection of primary human hepatocytes, though possible, has been subject to great variability (19). Recently, Gripon et al. (18) have reported that the addition of polyethylene glycol to HBV during exposure to cultured primary hepatocytes greatly enhances binding, internalization, and level of infection. It is possible that the polyethylene glycol added in vitro is substituting for another agent found in vivo. Attempts to demonstrate a physiological role for the apo H binding in HBV infection are in progress.

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